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Research Paper

Hypoglycemic, antihyperglycemic, and antioxidant effects of the edible plant *Anoda cristata* ☆

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ABSTRACT

Ethnopharmacological relevance: Some studies refer that the entire plant of *Anoda cristata* is consumed as food and medicine; in particular for treating diabetes, inflammation, fever, cough, and wounds. The aim of this study was to establish the preclinical efficacy of *Anoda cristata* as hypoglycemic and/or antihyperglycemic agent using well-known animal models.

Materials and methods: The acute toxicity was analyzed by the Lorke method. Acute hypoglycemic as well as oral glucose and sucrose tolerance tests were used to determine the hypoglycemic and antihyperglycemic action of *Anoda cristata*. Several preparations of the plant, including a mucilage (M), an aqueous (T-AE), a free mucilage aqueous (FM-AE), and an organic (OE) extracts, were tested in healthy and NA-STZ-hyperglycemic mice. Glibenclamide (15 mg/kg), acarbose (5 mg/kg) and metformin (200 mg/kg) were used as positive controls. The major compounds acacetin (**1**) and diosmetin (**2**), isolated from an infusion of the plant applying chromatographic methods, were evaluated as hypoglycemic agents using the same assays. The FM-AE was tested also in rats with metabolic syndrome induced by a high-fructose fed. Finally some assays were performed to determine the antioxidant capacity of the FM-AE *in vitro*.

Results: The results demonstrated that the extracts and compounds from *Anoda cristata* were effective for reducing blood glucose levels in healthy and NA-STZ-hyperglycemic mice when compared with vehicle groups ($p < 0.05$). The FM-AE exerted also positive effect over different biochemical parameters altered in rats with metabolic syndrome induced by a fructose diet. FM-AE has also antioxidant action effectively trapping $ONOO^-$ and $ROO^\bullet$ radicals. The major flavonoids isolated from the plant, namely acacetin (**1**) and diosmetin (**2**), caused significant hypoglycemic effect and possessed antioxidant activity.

Conclusion: Anoda cristata is effective to diminish glucose levels *in vivo* and to ameliorate different disorders related with the metabolic syndrome in rats. According to the results, the efficacy of *Anoda cristata* preparations could be due to the presence of active principles with different mode of actions at the molecular level, including α-glycosidases inhibitors, insulin secretagogues, glucose entrapment and radical trapping agents.

1. Introduction

Metabolic syndrome is a multiplex risk factor that arises from insulin resistance accompanying abnormal adipose deposition and function. It is a risk factor for coronary heart disease, as well as for diabetes, fatty liver, and several cancers. The underlying causes of metabolic syndrome include overweight and obesity, physical inactivity and genetic factors. According to the International Diabetes Federation (IDF), 382 million people of the world population suffer from diabetes, 90% of which are affected by type 2 diabetes mellitus. The incidence of this illness is increasing rapidly, and it is estimated that by 2035 this will rise to 592

Abbreviations: IDF, International Diabetes Federation; GLI, glybenclamide; STZ, streptozotocin; NA, nicotinamide; T-AE, total aqueous extract; FM-AE, free mucilage aqueous extract; M, mucilage; OP-AE, organic phase of free mucilage aqueous extract; OE, organic extract; OGTT, oral glucose tolerance test; OSTT, oral sucrose tolerance test; MS, metabolic syndrome

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million people. Type 2 diabetes mellitus is a metabolic disorder characterized by chronic high blood glucose levels, and alterations of carbohydrate, fat and protein metabolisms due to deficiencies in insulin secretion and/or action. The hyperglycemic condition promotes protein glycation, which in turn causes tissue damage and different complications including heart and peripheral vascular complaints, retinopathy, nephropathy and neuropathy.

There is no cure for type 2 diabetes mellitus, but the condition can be managed by changing patients lifestyle, and using treatments for keeping blood glucose level within the normal range to prevent the development of long-term effects. Oral medications are available to lower hyperglycemia, but these products possess side effects after prolonged use (Moller, 2001; Aicher et al., 2010; Tahrani et al., 2011). In consequence, the need for the search for new therapeutic options, including plants commonly consumed as food and medicine, has increased notably (Campbell-Tofte et al., 2012). Mexico is rich in edible medicinal plants highly prized by the population for the treatment of diabetes but only a few of these have been investigated for their preclinical or clinical efficacy (Andrade-Cetto and Heinrich, 2005; Alarcoń-Aguilar and Roman-Ramos, 2006; Mata et al., 2013). *Anoda cristata* (L) Schltdl. (Malvaceae) is among these species. According to Rendón et al. (2001), *Anoda cristata* grows in different environments throughout Mexico where it is commonly known as "alache", "quelite " (edible plant or herbal food), and field poppy. A few ethnobotanical studies refer the use of *Anoda cristata* as food. Thus, young, tender leaves and buds are consumed as "quelites" and can be eaten alone or combined with squash, beans, or corn (Rendón et al., 2001). The entire plant, including flowers, is used with other plants to treat stomach inflammation, fever, cough, and wounds. An infusion prepared from the dry aerial parts (a feast, ~10 g in 1 L of water) of the plant is also employed as drinking water for treating diabetes. In addition, the above ground plant is frequently used as forage for cattle. As other plants of the Malvaceae, *Anoda cristata* contains high amounts of mucilages (Rendón et al., 2001; Echevarría-Machado et al., 2005; Bautista-Cruz et al., 2011).

As part of our systematic investigation of Mexican medicinal flora as source of new alternative treatments for type 2 diabetes mellitus, in this work we describe the hypoglycemic, and anti-hyperglycemic action of *Anoda cristata* using different animal models.

2. Materials and methods

2.1. Reagents and solvents

Dichloromethane (CH_2Cl_2), methanol (MeOH), ethyl acetate (EtOAc), hexane, and ethanol, analytical or HPLC grade, were purchased from Merck, Darmstadt, Germany; acarbose, glibenclamide (GLI), metformin, nicotinamide (NA), streptozotocin 98% (STZ), sucrose, glucose, and Tween 80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercial fructose (Savien, KMD, Mexico City, Mexico) was employed.

2.2. Experimental animals and ethical aspects

The toxicity studies and antidiabetic tests were performed on male mice ICR (body weight range, 25–30 g); metabolic syndrome-induced rats were accomplished with male Sprague Dawley rats (body weight range 220–260 g). Both types of animals were purchased from Centro UNAM-Harlan (Harlan Laboratories SA de CV, Mexico). The animals were housed in groups of 10 mice or two rats under standard laboratory conditions (12 h light–dark cycle under controlled temperature, 22 ± 1 °C) and maintained on a standard pellet diet and water *ad libitum*. After the experiments, all animals were sacrificed in a CO_2 chamber. All experimental procedures involving animals and their maintenance were conducted in conformity with the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999) and in compliance with the international rules on care and use of laboratory animals. All efforts were made to minimize the number of animals used ($n=8$) as well as any discomfort. The animal protocols (FQ/CICUAL/085/14, and FQ/CICUAL/086/14) for this study were approved by the Institutional Animal Care and Use Committee at Facultad de Quimica-UNAM.

2.3. Plant material

The aerial parts of *Anoda cristata* were collected in Tepeaca (Puebla, Mexico) in November 2012. A plant specimen was authenticated by Professors Robert Bye and Edelmira Linares and deposited at the ethnobotanical collection of the National Herbarium (MEXU), UNAM, Mexico City (Voucher no: 2755).

2.4. Extracts preparation

The plant material was dried and grounded using a Thomas Willey laboratory mill (mesh size 2 mm). Dried and pulverized *Anoda cristata* plant material (50 g) was extracted with 2 L of boiling water during 30 min. After filtration, the infusion was evaporated to dryness under reduced pressure to give 6.0 g of total aqueous extract (T-AE). The procedure was repeated as needed. In order to obtain the free mucilage aqueous extract (FM-AE), the infusion was treated with ethanol in 1:1 ratio; the resulting precipitate (mucilage) was removed by centrifugation (10,000*g*; 20 min) (Bhatty, 1993), and the supernatant was evaporated to dryness under reduced pressure to give a free mucilage aqueous extract. For each 6 g of total aqueous extract, 4.75 g of FM-AE was obtained. The procedure was repeated as needed.

2.5. Isolation of acacetin (**1**) and disometin (**2**)

2.5.1. General experimental procedures

Melting points were determined using a Fisher-Johns apparatus and are uncorrected. NMR spectra were recorded using a Varian Unity Plus 400 spectrometer, at either 400 MHz (^1H) or 100 MHz (^{13}C) , in DMSO-d_6; tetramethylsilane (TMS) was employed as internal standard. Open column chromatography was carried out on silica gel 60, 70–230 mesh (Merck, Darmstadt, Germany). Thin layer chromatography analyses were performed on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany), and visualization of the plates was carried out using a ceric sulfate (10%) solution in H_2SO_4.

2.5.2. Isolation and characterization of compounds **1** and **2**

170 g of FM-AE were re-suspended in distilled water and partitioned with CH_2Cl_2 (1 L × 3) and EtOAc (1 L × 3). The organic phases were combined and concentrated *in vacuo* to yield a brown residue (OP-AE, 2.152 g). 400 mg of which were subjected to open column chromatography on silica gel, and eluting with a gradient of hexane–EtOAc (10:0→0:10) and EtOAc–MeOH (10:0→0:10) to yield 17 secondary fractions (F_1–F_{17}). From fractions F_8 and F_{11} crystallized 20 mg of acacetin (**1**) and 25 mg of diosmetin (**2**), respectively, which were the two major components of OP-AE (Fig. S1). The mother liquors were not further processed. The structures of compounds **1** and **2** (Fig. 1) were established by spectroscopic and spectrometric analyses and compared with literature data (Wawer and Zielinska, 2001; Park et al., 2007).

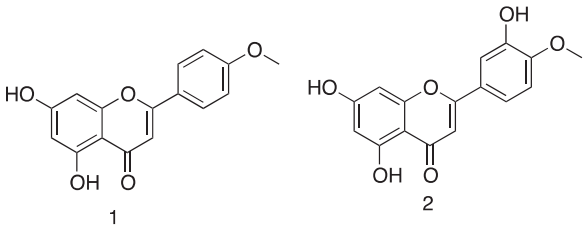

Fig. 1. Chemical structures of acacetin (**1**) and diosmetin (**2**).

2.6. Acute toxicity assay

Mice were divided into control and test groups ($n=3$) and treated in two phases according to the Lorke protocol. In the first one, the control group and the test groups received intragastric doses of vehicle, 10, 100 and 1000 mg/kg of T-AE, FM-AE, M or OE. In the second, the animals received doses of 1600, 2900 and 5000 mg/kg of the same treatments. In each phase, mice were observed daily during 14 days for mortality, toxic effects and/or changes in behavioral pattern. At the end of the each phase the animals were sacrificed in a CO_2 chamber and the main organs (liver, heart, lung, spleen and kidneys) were observed macroscopically and compared with those of the control group (Lorke, 1983).

2.7. Pharmacological evaluations

2.7.1. Preparation of test samples

All treatments (T-AE, FM-AE, M, OE and compounds **1** and **2**) were suspended in saline solution with 0.05% Tween 80 (vehicle) for oral administration (*po*). As a hypoglycemic positive control, GLI was used at the dose of 15 mg/kg. Acarbose was employed as an antihyperglycemic drug at the dose of 5 mg/kg. Glucose (1 g/kg) and sucrose (3 g/kg) were used as carbohydrates to carry out the glucose and sucrose tolerance tests, respectively.

2.7.2. Induction of hyperglycemia in mice, acquisition of blood samples and measurement of blood glucose levels

To induce a hyperglycemic condition in mice, first an *ip* administration of NA (50 mg/kg) dissolved in saline solution was used (Masiello et al., 1998). 15 min later a single intraperitoneal injection (*ip*) of freshly prepared STZ (130 mg/kg) dissolved in 0.1 M citrate buffer, pH 4.5 was administered. One week later, mice with blood glucose levels higher than 150 mg/dL were considered hyperglycemic and included in the study. Blood samples were obtained from the caudal vein by means of a small incision at the end of the tail. A commercial glucometer One Touch Ultra 2 (Johnson & Johnson, Milpitas, CA, USA) was used to estimate the blood glucose levels (mg/dL) by the enzymatic glucose oxidase method. For each treatment was calculated the percentage variation of glycemia with respect to the initial (0 h) level, according to the following equation: % variation of glycemia $=[(G_t-G_i)/G_i]\times 100$, where G_i is initial glycemia value and G_t is the glycemia value after treatment administration (Escandón-Rivera et al., 2012).

2.7.3. Acute hypoglycemic assay

Healthy or NA-STZ-hyperglycemic mice were treated with T-AE, FM-AE, M (31.6, 100, and 316 mg/kg of body weight), **1** or **2** (3, 10 and 31.6 mg/kg of body weight). Blood samples were collected at 0, 1.5, 3, 5, 7, and 9 h after treatment administration. GLI (15 mg/kg, *po*) was used as positive control and saline solution as vehicle control. The hypoglycemic response was evaluated using the total area under the curve by the trapezoidal method (Escandón-Rivera et al., 2012).

2.7.4. Oral glucose tolerance test (OGTT)

Groups of eight healthy or NA-STZ-hyperglycemic mice were placed in acrylic boxes. All groups were put on fast during 4 h before the experiments, with water *ad libitum*. The preparations (T-AE, FM-AE, M) from *Anoda cristata* were tested at doses of 31.6, 100, and 316 mg/kg of body weight, *po*; metformin (200 mg/kg) was suspended in the same vehicle. Time 0 min was set before treatment with the extract or compounds; 30 min later a glucose load (1 g/kg) was administered *po* to the animals. Blood samples were obtained 30, 60, 90, 120, and 180 min, and the % variation of glycemia was calculated.

2.7.5. Oral sucrose tolerance test (OSTT)

The assay was performed using the same methodology described for the OGTT but using sucrose (3 g/kg of body weight) as the carbohydrate. In this experiment the extracts (T-AE, FM-AE, M and OE) were tested in healthy and hyperglycemic animals. In both cases the doses were 31.6, 100 and 316 mg/kg or 31.6, 56.2 and 100 mg/kg for OE. As positive control acarbose was used (5 mg/kg, *po*). The % variation of glycemia was calculated for all treatments.

2.7.6. Induction of metabolic syndrome (MS) in Sprague Dawley rats

After adaptation during seven days, rats were randomly divided into four groups ($n=8$) and housed as couples in acrylic cages. Rats were fed with regular rodent chow (2018S pellets: 44.2% carbohydrate, 18% protein, 6.2% fat; 3.1 kcal/g; Teklad Global Diets, Harlan Laboratories Inc., Indianapolis, IN, USA). In addition, group I (vehicle control) received water, and groups II–IV were fed with regular rodent chow and 10% fructose solution as drinking water. The fructose solution was administered throughout the experiment. The animals were weighed and measured (nose to anus length) at the beginning and at the end of the experiment period; Lee index was calculated dividing the cubic root of the final body weight (g) by the final body length (cm) and multiplying by 1000. The period for inducing metabolic syndrome was 12 weeks (Ferreira de Moura et al., 2009). The bottles of water or fructose solution were refilled each 2 days. The differences between the full bottles and the content 48 h later were considered as the amount of fructose solution ingested during the period.

2.7.6.1. Oral glucose tolerance test in MS-induced rats. OGTT was conducted as previously described. Fasting (4 h) 12 weeks metabolic syndrome-induced rats were divided in groups ($n=8$). Group I was used as vehicle control group, group II as fructose control group both received vehicle (saline solution, *po*) and groups III and IV were treated with FM-AE (100 mg/kg and 316 mg/kg, *po*; 0.2 mL/100 g bw). Basal glycemia was recorded before intragastric administration of treatments; 30 min later all animals received a glucose load (2 g/kg bw, *po*). Blood samples were collected at 30, 60, 90, and 120 min after glucose load. Percentage variation of glycemia was calculated as previously described for mice.

2.7.6.2. Effects of daily treatment with FM-AE in MS-induced rats. After the OGTT, the animals were allowed to recover during six days while receiving water or fructose solution in their diet. Week 14 (day 98) animals from groups III and IV were treated during seven days with free mucilage dry aqueous extract (FM-AE) of *Anoda cristata* (100 mg/kg and 316 mg/kg, *po*, $n=8$) while maintaining the fructose administration diet. Group I was treated with vehicle (*po*), water and normal diet, whereas group II with vehicle (*po*) and fructose solution as drinking water till day 104.

2.7.6.3. Collection of blood and liver samples. At day 105, all animals were fasted for 4 h, with free access to water, and anesthetized with an intramuscular injection of ketamine–xilazine (80–10 mg/kg) to obtain blood with retro-orbital puncture. The serum was separated of blood by centrifuging (3000*g*, 5 min). After collecting blood samples, livers were excised immediately. All serum and tissue samples were stored at −80 °C until use for determination of biochemical parameters.

2.7.6.4. Biochemical parameters determination. The serum total cholesterol, triglycerides, uric acid and glucose levels were determined using colorimetric reactions with commercial kits (RANDOX Laboratories, Antrim, United Kingdom).

2.7.6.5. Measurement of hepatic glycogen content. Hepatic glycogen content was analyzed according to the procedure of Murat and Serfaty, 1974. Briefly, 50 mg of liver samples was homogenized in 1 mL of ice-cold citrate buffer (0.1 M, pH 4.2). Homogenates were diluted with citrate buffer to obtain a concentration of 5 mg/mL. 10 μL of this dilution was mixed with 10 μL of amyloglucosidase (exo-1,4-α-glucosidase, EC 3.2.1.3; 1 mg/mL, Sigma-Aldrich). The mixture was allowed to stand at room temperature for 2 h. A sample (10 μL) was used to determine free glucose using a Trinder GOD–POD method (Spinreact, Spain).

2.7.6.6. Serum insulin levels. The insulin levels were estimated using the Rat Insulin Enzyme Immunoassay Method (ALPCO Diagnostics, Salem, NH, USA).

2.8. In vitro antioxidant activity

2.8.1. $O_2^{\bullet -}$ scavenging assay

The xanthine–xanthine oxidase system was used to determine the $O_2^{\bullet -}$ scavenging capacity of the extract. Generation of $O_2^{\bullet -}$ and xanthine oxidase activity were measured as the reduction of nitroblue tetrazolium (NBT at 560 nm) and uric acid (at 295 nm) production, respectively, by using a spectrophotometer (DU-640 series Beckman) as previously described (Gaona-Gaona et al., 2011). Nordihydroguaiaretic acid was used as standard (Fontana et al., 2001). All tests were performed in triplicate. The results were expressed as percent of $O_2^{\bullet -}$ scavenging capacity of FM-AE (40–4000 μg).

2.8.2. $^{\bullet}OH$ scavenging capacity

The ability of FM-AE to scavenge hydroxyl radical ($^{\bullet}OH$) was assessed using the Fe^{3+}–EDTA–H_2O_2–deoxyribose system following a well described method (Floriano-Sanchez et al., 2006); $^{\bullet}OH$ was generated by Fe^{3+}–EDTA–H_2O_2 reaction and terephthalic acid (TA) was used to assess the radical generation (Gaona-Gaona et al., 2011). The extent of deoxyribose degradation by the formed $^{\bullet}OH$ was measured directly in the aqueous phase by the thiobarbituric acid (TBA) test measuring the optical density at a wavelength of 532 nm using a Genesys 8 spectrophotometer. Dimethylthiourea was used as standard for $^{\bullet}OH$ scavenging.

2.8.3. Peroxynitrite ($ONOO^-$) scavenging capacity

Peroxynitrite ($ONOO^-$) scavenging activity was executed as previously described (Floriano-Sanchez et al., 2006) by monitoring fluorescein formation from 2′,7′-dichlorodihydro-fluorescein diacetate (DCF). DL-penicillamine was used as reference compound.

2.8.4. H_2O_2 scavenging assay

The scavenging capacity was evaluated applying the method described by Long et al. (1999) using the FOX reagent (xylenol orange, ammonium ferrous sulfate and H_2SO_4). The concentration of H_2O_2 was recorded at 560 nm. Sodium pyruvate was used as standard.

2.8.5. Singlet oxygen scavenging assay

The production of singlet oxygen ($^1O_2$) by sodium hypochlorite and H_2O_2 was determined using a spectrophotometric method (Medina-Campos et al., 2007). The $^1O_2$ scavenging assay is based on N,N-dimethyl-p-nitrosoaniline (DMNA) bleaching in the presence of $^1O_2$. The extent of $^1O_2$ production and the effect of extract on it were determined by measuring the decrease in the absorbance of DMNA at 440 nm. The relative scavenging efficiency (% inhibition in production of $^1O_2$) was estimated from the difference in absorbance of DMNA with and without the addition of increasing amounts of FM-AE. Glutathione was used as a reference.

2.8.6. Hypochlorous (HOCl) scavenging assay

For HOCl scavenging assay, the catalase method was utilized. The peak of catalase disappeared at 404 nm with HOCl, indicating protein oxidation. Experiments were carried out as previously described (Aruoma and Halliwell, 1987). Lipoic acid was used as reference compound.

2.8.7. Peroxyl radical ($ROO^{\bullet}$) scavenging assay

For $ROO^{\bullet}$ scavenging the ORAC fluorometric assay was used (Huang et al., 2002). In this assay, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was used as peroxyl radical generator; Trolox was used as standard and fluorescein was used as fluorescent probe. Fluorescence was read each minute using an excitation wavelength of 485 nm and an emission wavelength of 520 nm using a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VA, USA). The change of fluorescence intensity is an index of the degree of free radical damage.

2.9. Statistical analysis

The data obtained were analyzed by the Prism program Version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the means ± SEM of 8 animals in each group. To determine statistically significant differences between groups an ANOVA (one or two ways) was used followed by Dunnett's or Bonferroni *post-hoc* tests; $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Acute toxicity study in mice

Since *Anoda cristata* is a weed frequently employed by humans as medicine and food, the first step in this investigation was to assess the potential acute toxic effect in mice of a few extracts of the aerial parts of the plant (T-AE, FM-AE, M and OE). For this endeavor, the Lorke procedure was employed (Lorke, 1983). The different treatments did not provoke animal death, behavioral alterations, lesions, or bleeding of the internal tissues and organs of the animals. Thus, none of the extracts tested were toxic according to the Lorke criteria. These results are consistent with the safe consumption of this edible green indigenous species, which according to a previous study possesses high protein content (Rendón et al., 2001; Bautista-Cruz et al., 2011).

3.2. Hypoglycemic and antihyperglycemic effects in mice of Anoda cristata extracts

In order to establish the potential of *Anoda cristata* for treating diabetes, different assays were performed using a well-known

A-STZ-hyperglycemic mice model (Masiello et al., 1998). Experimental induction of type 2 diabetes was achieved by treating mice with streptozotocin (STZ, 130 mg/kg), 15 min after an injection of nicotinamide (NA, 50 mg/kg). The treatment with NA provokes partial protection against the cytotoxic action of STZ by scavenging free radicals. In turn, this action causes minor damage to pancreatic β-cells creating a hyperglycemic condition similar to type 2 diabetes (Mythili et al., 2004). The doses of NA and STZ used were in the range usually reported to induce a hyperglycemic condition in different experimental animals (Fröde and Medeiros, 2008).

Oral administration of T-AE, FM-AE, M (31.6, 100 and 316 mg/kg) or OE (31.6, 56.2 and 100 mg/kg) caused significant reduction in blood glucose levels in healthy (Fig. S2) and NA–STZ treated (Fig. 2) mice when compared with vehicle-treated group. The best hypoglycemic effect was observed with FM-AE (Fig. 2B); on the other hand, M showed better hypoglycemic action than T-AE (Fig. 2C and A). The results for M are in agreement with the beneficial effect of mucilages for ameliorating blood glucose levels in hyperglycemic rodents and humans (Nuñez-Lopez et al., 2013; Rodríguez-Morán et al., 1998; Kumar et al., 2005). Mucilages are naturally occurring, high molecular weight polysaccharides consisting of sugars and uronic acid units (Singh et al., 2009). In a recent paper, Nuñez-Lopez et al. (2013) suggested that the glucose lowering effect of these products is associated with a glucose entrapment mechanism, due to the viscosity and swelling properties of these substances.

Next, T-AE, FM-AE, and M (31.6, 100 and 316 mg/kg) were assayed using an OGTT; the results (Figs. 3–5) indicated that they lowered blood glucose levels in healthy and hyperglycemic mice. Those effects can be only explained in terms of an inhibitory action of glucose transport and/or with a glucose entrapment mechanism in the case of M (Nuñez-Lopez et al., 2013). The extracts (31.6, 100 and 316 mg/kg for T-AE and M; and 31.6, 56.2 and 100 mg/kg for OE) were tested also by means of an OSTT. This test is performed usually to assess if the antihyperglycemic effect of a drug implicates inhibition of intestinal α-glycosidases; these enzymes catalyze carbohydrate breakdown into monosaccharides in order to facilitate their absorption. The results illustrated in Figs. 6–8 revealed that all preparations tested were antihyperglycemic in healthy and hyperglycemic mice; in this case the rate of glucose absorption into the bloodstream was reduced. Therefore, an inhibitory effect on intestinal α-glycosidases might be involved in the antihyperglycemic action of the extracts. The lower effect displayed by the mucilage in the OSTT, in particular in diabetic animals, suggests a glucose trap mechanism. Altogether, the results revealed that *Anoda cristata* extracts possess significant antihyperglycemic and hypoglycemic actions.

3.3. Hypoglycemic effect of compounds **1** and **2**

Next, the two major flavonoids of the aqueous extract (Fig. S1), namely acacetin (**1**) and diosmetin (**2**), were isolated,

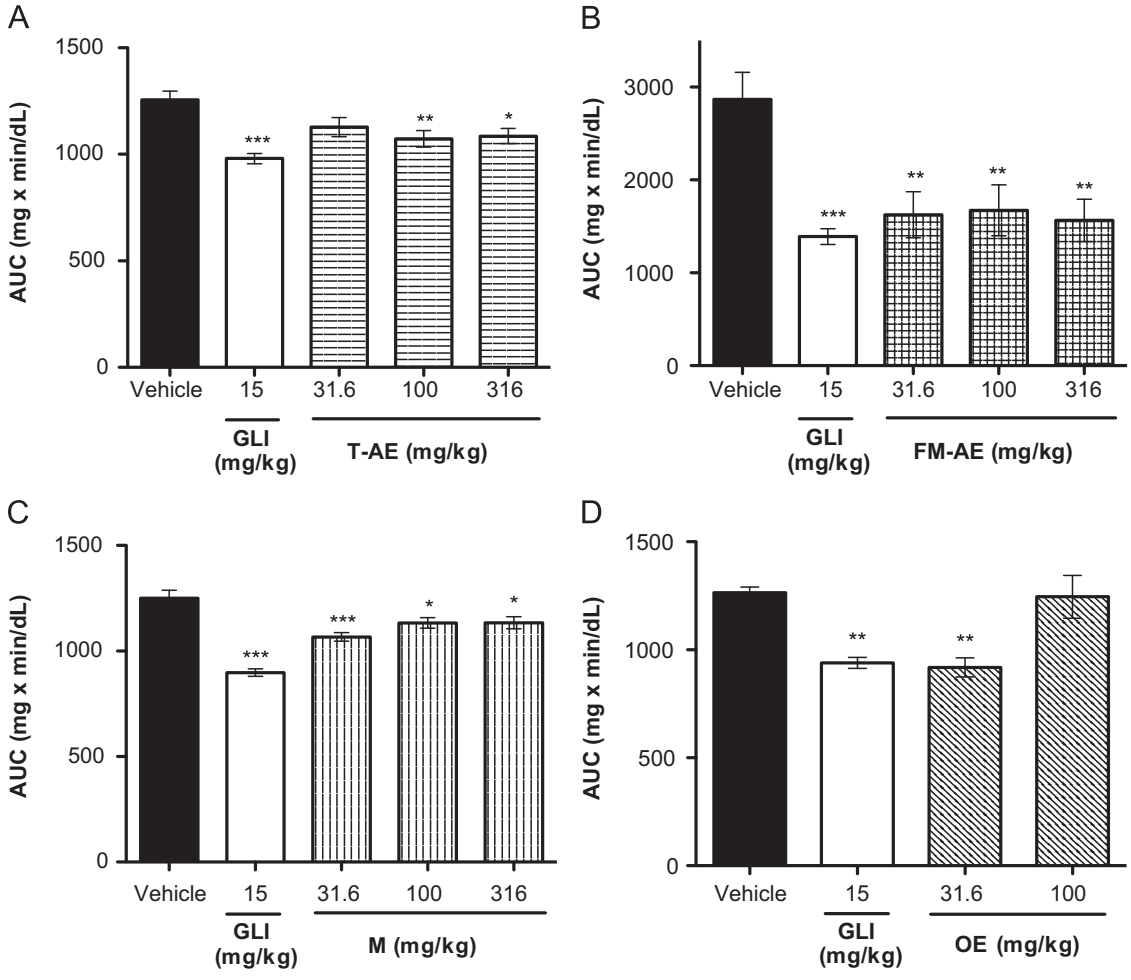

Fig. 2. Hypoglycemic actions of (A) T-AE, (B) FM-AE, (C) M and (D) OE in NA–STZ hyperglycemic mice. Bars represent the mean for $n=8$ mice. Data were analyzed by ANOVA and *post-hoc* Dunnett's test. *, **, *** denote significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* vehicle group.

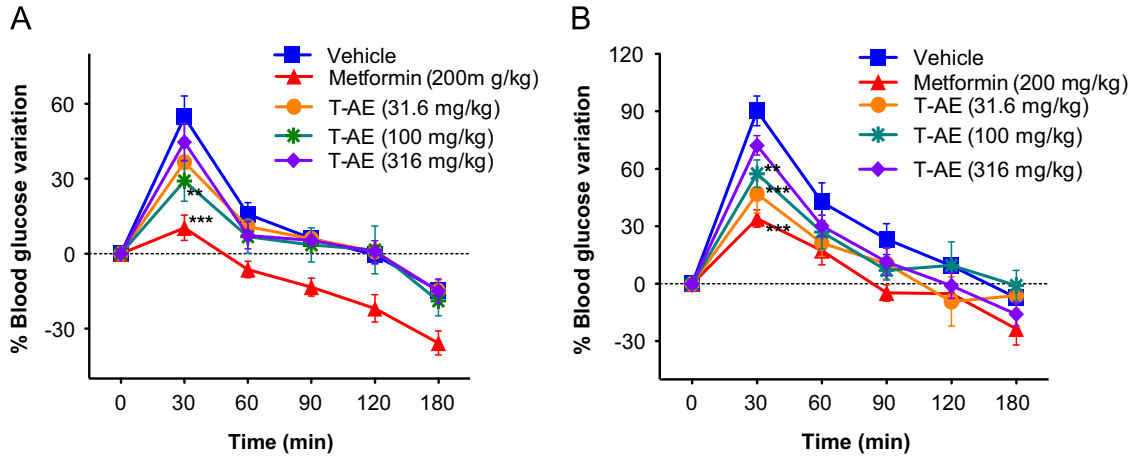

Fig. 3. Influence of the T-AE on postprandial blood glucose levels in healthy (A) and NA–STZ hyperglycemic (B) mice during an OGTT. Values are presented as the mean for $n=8$ mice. Data were analyzed by ANOVA and *post-hoc* Bonferroni test. *, **, *** denote significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* vehicle group.

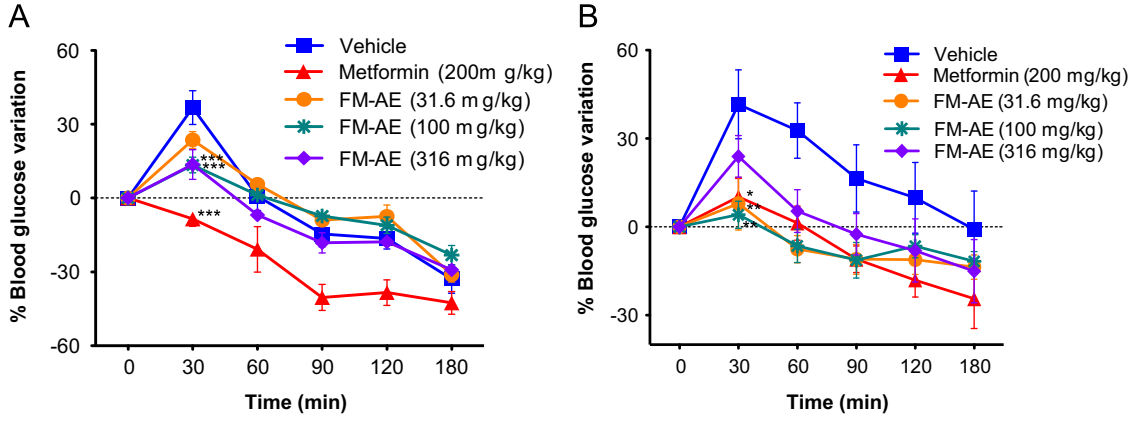

Fig. 4. Influence of FM-AE on postprandial blood glucose levels in healthy (A) and NA–STZ hyperglycemic (B) mice during an OGTT. Values are presented as the mean for $n=8$ mice. Data were analyzed by ANOVA and *post-hoc* Bonferroni test. *, **, *** denote significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* vehicle group.

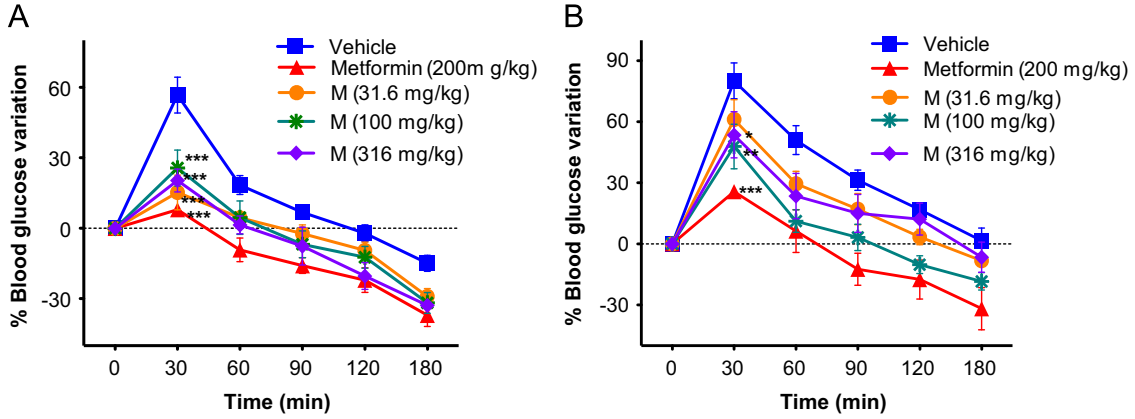

Fig. 5. Influence of M on postprandial blood glucose levels in healthy (A) and NA–STZ hyperglycemic (B) mice during an OGTT. Values are presented as the mean for $n=8$ mice. Data were analyzed by ANOVA and *post-hoc* Bonferroni test. *, **, *** denote significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* vehicle group.

characterized and tested in an acute hypoglycemic assay. Compound **1** (3 and 31.6 mg/kg) caused significant decrease in blood glucose levels in healthy and hyperglycemic mice when compared with vehicle-treated groups; compound **2** (3, 10 and 31.6 mg/kg) was active at all doses tested in healthy mice, but in hyperglycemic animals was active only at the lower dose (Figs. 9 and 10). Both compounds have previously shown biological activities related with their antidiabetic potential. Thus, a few diosmetin glycosides induced a decrease in blood glucose, cholesterol and triglycerides levels in diabetic rats; these glycosides provoked also an increment of glutathione peroxidase and superoxide dismutase activities in liver (Michael et al., 2013). In other studies, it was found that plant extracts containing diosmetin and acacetin were insulinotropic and antihyperglicemic (Stefkov et al., 2011). Furthermore, the antiglycation activity induced by an *Artocarpus heterophyllus* extract has been attributed to its content of diosmetin (**2**) and other flavonoids (Deve

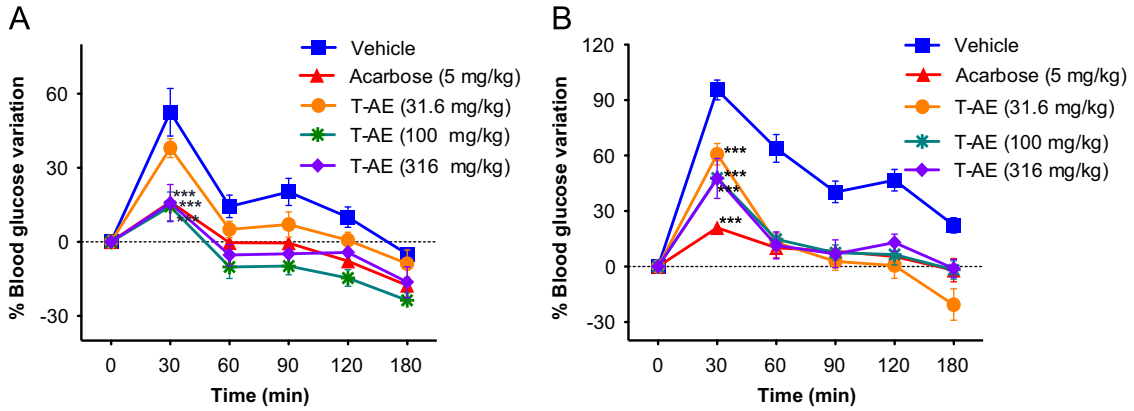

Fig. 6. Influence of T-AE on postprandial blood glucose levels in healthy (A) and NA–STZ hyperglycemic (B) mice during an OSTT. Values are presented as the mean for $n=8$ mice. Data were analyzed by ANOVA and *post-hoc* Bonferroni test. *, **, *** denote significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* vehicle group.

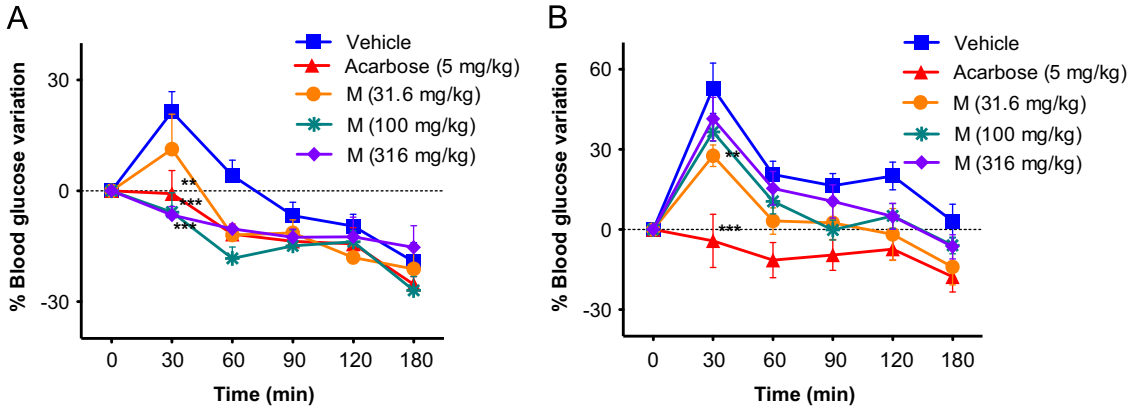

Fig. 7. Influence of M on postprandial blood glucose levels in healthy (A) and NA-STZ hyperglycemic (B) mice during an OSTT. Values are presented as the mean for $n=8$ mice. Data were analyzed by ANOVA and *post-hoc* Bonferroni test. *, **, *** denotes significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* vehicle group.

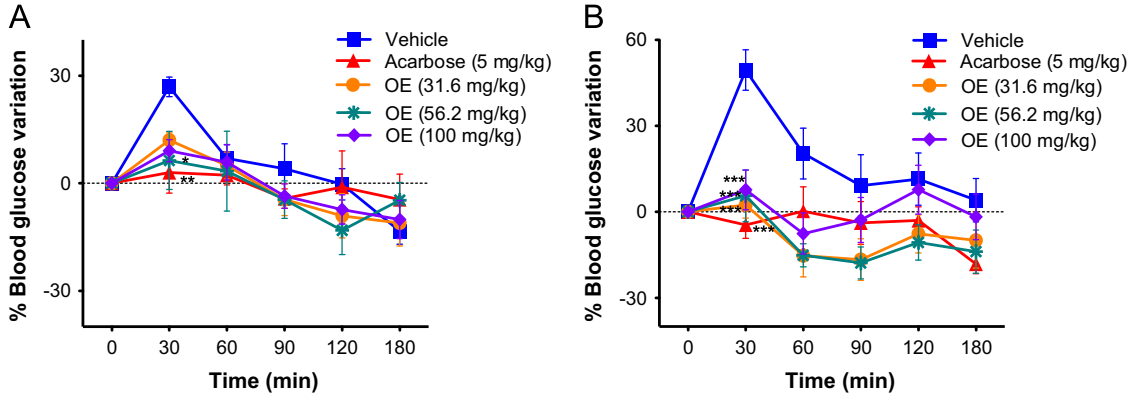

Fig. 8. Influence of OE on postprandial blood glucose levels in healthy (A) and NA–STZ hyperglycemic (B) mice during an OSTT. Values are presented as the mean for $n=8$ mice. Data were analyzed by ANOVA and *post-hoc* Bonferroni test. *, **, *** denote significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* vehicle group.

et al., 2014). Acacetin and its glycosides exhibited also significant inhibitory activity against α-glycosidases (Escandón-Rivera et al., 2012; Ezzat and Salama, 2014) while acacetin (**1**) and diosmetin (**2**) behaved as PPAR agonists *in vitro* (Matin et al., 2009, 2013).

3.4. *Effect of day treatment of FM-AE on fructose induced MS rats*

Considering the good hypoglycemic and antihyperglycemic action of *Anoda cristata* preparations, the study was extended to assess the effect of FM-AE on a series of biochemical parameters usually altered in individuals with metabolic syndrome. Diagnosis of this metabolic alteration implies positive results to at least three signs or symptoms, namely, insulin resistance, glucose intolerance, hypertension, obesity, and blood lipid profile variations. Then, a set of experiments with rats fed with a fructose diet was performed. Rats fed with fructose develop these metabolic impairments (Ferreira de Moura et al., 2009; Kim et al., 2010, Cheng et al., 2014) because fructose does not stimulate insulin secretion from pancreatic β-cells, thus provoking a decrement of leptin, appetite increment and weight gain (Elliott et al., 2002; Basciano et al.,

2005). Furthermore, according to some authors (Ferreira de Moura et al., 2009), a high flux of fructose to the liver perturbs glucose metabolism, glucose uptake pathways, and stimulates lipogenesis and triglycerides accumulation. In consequence, reduced insulin sensitivity, hepatic insulin resistance and glucose intolerance are observed (Basciano et al., 2005; Ferreira de Moura et al., 2009).

The results of the experiments with rats fed with a fructose diet for FM-AE are summarized in Table 1. The data revealed important differences between controls (vehicle or fructose 10%) and FM-AE treated animals. Although no significant changes in body weight were observed, the rats fed with fructose exhibit a greater Lee index than those treated with FM-AE (316 mg/kg). The Lee index for rats is equivalent to the human body mass index; indirectly, this parameter measures body fat and the degree of overweight or obesity (Basciano et al., 2005; Kim et al., 2010). On the other hand, compared with control-vehicle rats, the fat depot was higher in the high fructose-fed rats. This parameter was considerably reduced in the FM-AE treated groups. The high levels of serum glucose, triglycerides and uric acid observed in fructose-fed rats also decreased significantly in the groups treated with FM-AE. Moreover, the group treated with 316 mg/kg of FM-AE exhibited significantly higher insulin levels than those in both control groups (groups I and II). Finally, no significant differences in serum total cholesterol and glycogen levels were detected between treated and non-treated animals. Thus, treatment with FM-AE decreased fat depot weight and Lee index in fructose induced MS rats due to an increment in insulin secretion and a reduction of lipogenesis. On the other hand, this preparation seems to decrease the production of uric acid after a load of fructose. If these effect is related to a decrease in the activity of the enzyme xanthine oxidase, usually increase upon fructose consumption, remains an open question.

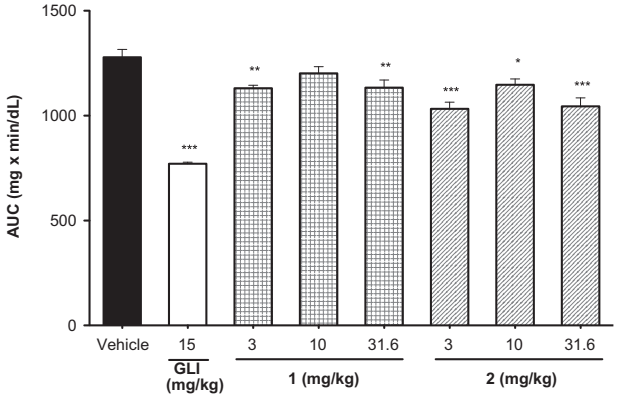

Fig. 9. Hypoglycemic effect of compounds **1** and **2** in healthy mice. Bars represent the mean for $n=8$ mice. Data were analyzed by ANOVA and *post-hoc* Dunnett's test. *, **, *** denote significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* vehicle group.

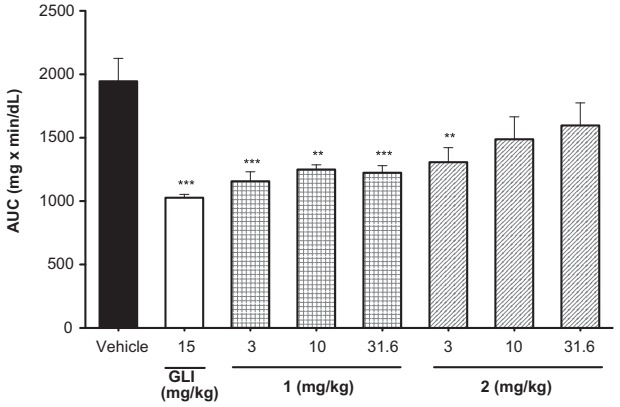

Fig. 10. Hypoglycemic effect of compounds **1** and **2** NA-STZ hyperglycemic mice. Bars represent the mean for $n=8$ mice. Data were analyzed by ANOVA and *post-hoc* Dunnett's test. *, **, *** denotes significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* vehicle group.

Glucose intolerance represents other important symptom in the metabolic syndrome therefore FM-AE was evaluated to assess its ability to improve glucose tolerance after an OGTT. The results in Table 2 indicate that FM-AE suppressed significantly the post-prandial peak 30 min after the oral administration of the carbohydrate load, at the two doses tested; these results are shown graphically in Fig. S3. Thus, FM-AE could ameliorate glucose intolerance in individuals affected with metabolic syndrome.

3.5. *In vitro antioxidant activity*

Oxidative stress is considered the unifying factor in the development of diabetes complications (Sakai et al., 2003; Houstis et al., 2006; Bashan et al., 2009; Afanas'ev, 2010; Bagul et al., 2012). Changes in oxidative stress, either steaming from glucose mediated increase free radical generation and/or reduction of endogenous antioxidant, are strong contenders for the title of root cause of diabetic complications. Therefore, the regulation of damaging ROS and RSN levels in diabetes by antioxidants and free radical scavengers represents an attractive benefit during the treatment of type 2 diabetes mellitus. In this context, FM-AE was evaluated as potential antioxidant and free radical scavenger agent following standard protocols (Medina-Campos et al., 2007; Gaona-Gaona et al., 2011).

FM-AE was only able to scavenged $ROO^{\bullet}$ (Fig. S4; FM-AE $IC_{50}=3.72\pm0.06$ μg/mL) radical, which attack all types of biological molecules and has been detected in diabetes, Alzheimer's

Table 1
Effects of FM-AE from *Anoda cristata* on fructose-fed rats with metabolic syndrome.

Parameter	Control (vehicle)	Control (fructose 10%)	FM-AE (100 mg/kg)	FM-AE (316 mg/kg)
Lee index	295.52 ± 2.18^{c}	314.82 ± 2.56	308.45 ± 1.74	307.91 ± 1.32^{a}
Body weight (g)	387.33 ± 4.37	446.67 ± 20.43	427.33 ± 8.19	428.33 ± 26.31
Fat depot weight (g)	8.26 ± 0.73^{c}	14.36 ± 0.55	10.19 ± 0.54^{c}	8.70 ± 0.49^{c}
Serum insulin (ng/mL)	1.02 ± 0.15^{a}	0.56 ± 0.05	0.67 ± 0.06	1.10 ± 0.15^{a}
Serum glucose (mmol/L)	6.72 ± 0.20^{b}	7.82 ± 0.27	6.85 ± 0.24^{a}	6.18 ± 0.16^{c}
Serum total cholesterol (mmol/L)	2.49 ± 0.02	2.55 ± 0.07	2.69 ± 0.09	2.62 ± 0.12
Serum triglycerides (mmol/L)	0.81 ± 0.05^{c}	1.71 ± 0.04	1.50 ± 0.09	1.37 ± 0.07^{b}
Serum uric acid (mmol/L)	0.18 ± 0.016	0.23 ± 0.021	0.174 ± 0.005	0.203 ± 0.013
Glycogen (mg/liver mg)	38.26 ± 6.10^{a}	24.24 ± 2.02	17.83 ± 0.78	24.30 ± 1.21

Values are reported as the mean ± SE for $n=8$ rats. Data were analyzed by ANOVA and p*ost-hoc* Dunnett's test. Letters a–c denote significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* control fructose 10% group.

Table 2
Effects of FM-AE from *Anoda cristata* on fructose-fed rats with metabolic syndrome during an OGTT.

Test samples	Initial glycemia (mg/dL)	% blood glucose variation			
	0 min	30 min	60 min	90 min	120 min
Vehicle	90.33 ± 2.82 (0%)	55.54 ± 7.59[c]	46.74 ± 11.20	28.40 ± 13.41	18.01 ± 12.20
Control (fructose 10%)	91.17 ± 3.97 (0%)	134.14 ± 34.31	80.91 ± 15.45	33.79 ± 10.72	26.24 ± 11.15
FM-AE (100 mg/kg)	88.33 ± 3.54 (0%)	62.38 ± 2.42[c]	39.76 ± 4.31	22.73 ± 6.68	7.70 ± 5.73
FM-AE (316 mg/kg)	91.67 ± 3.86 (0%)	48 0.97 ± 3.83[c]	39.58 ± 4.97	32.45 ± 10.32	24.14 ± 8.98

Values are reported as the mean ± SE for $n=8$ rats. Data were analyzed by ANOVA and *post-hoc* Bonferroni test. Letters a–c denote significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) *vs* control fructose 10% group.

disease, atherosclerotic tissues of patients with cardiovascular diseases, as well as during cell apoptosis and DNA-damage (Spiteller, 2008).

In conclusion, the information generated in this study indicates that the consumption of *Anoda cristata* is effective *in vivo* to ameliorate a hyperglycemic condition. Our data suggest that the efficacy of *Anoda cristata* preparations could be due to the presence of compounds with different targets at the molecular level, including α-glycosidases inhibitors, glucose transport enhancers and/or glucose entrapment agents, secretagogues, leptin and lipogenesis regulators. The presence of acacetin (**1**) and diosmetin (**2**), which are PPAR agonists and antioxidant agents, partially accounts for the observed effects (Matin et al., 2009, 2013). Taken together, these results indicate that *Anoda cristata* represents a valuable phytotherapeutic agent for treating type 2 diabetes mellitus. This work will be also useful for creating food composition databases including information of those edible plants useful for lowering the risk of type 2 diabetes and cardiovascular diseases.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.11.052.

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